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Endophytic fungi in *Quercus cerris*: isolation frequency in relation to phenological phase, tree health and the organ affected

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Summary. The isolation frequency of endophytic fungi in *Quercus cerris* was determined in relation to sampling data, the health state of the tree, and the tree organ affected. Sampling was carried out at three times, in April (buddbreak), June (full vegetation) and October (leaf fall), on trees that were either healthy or in decline, and on three organs: current-year twigs, with a diameter less than 2 cm, buds, and leaves. The experiment was done in an approximately 20-year-old oak forest at an altitude of 350–400 m above sea level. Three healthy and three declining trees were sampled annually in 1999 and 2000. From each tree 20 current-year twigs, 20 buds and 10 leaves were harvested. Tissue fragments, after sterilization with 10% H₂O₂, were incubated on potato-dextrose-agar amended with streptomycin, at 20°C in the dark for 7 days, and the isolated mycelia maintained at 4°C on malt-extract agar. A total of 15 fungal species were isolated. All organs yielded fungal species but the greatest number of endophytic species came from current year twigs. Individual species were isolated more often from declining trees than from healthy trees, and more fungi were isolated in June (full vegetation) than in April or October. *Diplodia mutila*, *Discula quercina* and *Phomopsis quercina*, which are known to be pathogenetic on oaks, were present on oaks for a large part of the year. These fungi are both saprophytes (as endophytes) and parasites and are, therefore, probably a serious contributing and inciting factor in the oak decline syndrome.

Key words: endophytic fungi, *Quercus cerris*, isolation frequency.

Introduction

The forest ecosystem, like ecosystems generally, is characterised by the coexistence in a certain time and space of a number of living components that establish between them mutualistic, neutral or antagonistic relations at various levels (Bernstein and Carroll, 1977; Carroll *et al.*, 1977; Grif-

fith and Boddy, 1988; Petrini and Fisher, 1988; Aoki *et al.*, 1990; Baird, 1991; Bills and Polishook, 1991; Kowalski and Kehr, 1997).

The relations within and between micro-organisms and trees in a forest ecosystem, certainly less degraded than those in an agricultural system, are in a dynamic state of evolution. Over the long term the relations between plants and micro-organisms give rise to durable states of coexistence that may produce a true endemic equilibrium.

The plant pathologist is well aware that endemicity is a state of equilibrium in which over time some of the component factors may evolve in-

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dipendently, resulting in the development of lethal epidemics.

Among the micro-organisms that have relations with the higher plants must be numbered the endophytic fungi, which sometimes form communities that are specific to a certain host and a certain environment (Petrini, 1996).

Endophytic fungi include some weakness parasites that become more virulent only when their host is weakened by stress. The plant under stress is then attacked by the endophyte and is invariably killed by it. It is precisely the weakness parasites living as endophytes that may become pathogenic as the physiological state of the trees deteriorates, and thus break the state of endemic equilibrium (Butin and Kowalski, 1983a, 1983b, 1990; Kowalski and Butin, 1989).

Research in the last few years has focussed on weak parasites having an endophytic behaviour in an attempt to shed light on the decline that is affecting various tree species, especially broadleaves, all over Europe, in Japan and in the United States (Griffin and Manion, 1985; Petrini, 1991; Halm-schlager, 1992).

In Italy, where oak stands are severely affected by decline, studies have been carried out since 1980 (Ragazzi *et al.*, 1989; Cellerino *et al.*, 1991; Grana-ta and Agosteo, 1991; Luisi *et al.*, 1991) on a number of endophytes and their possible role in the death of various oak species (Vannini and Anselmi, 1997; Ragazzi *et al.*, 1999a).

Studies conducted in Italy on the weak parasites causing the final stages of decline in many forest tree species, particularly oaks, and which generally are also, in a series of adverse circumstances, the factor bringing about the death of the tree, show that they go through an endophytic stage without symptoms in the host tissue, which then appears healthy.

The scarce knowledge of the biology and epidemiology of some endophytes that have been already isolated, or others that remain to be discovered, yet, has prompted this investigation into the endophytes colonising various organs of *Q. cerris* L., and in particular *Discula quercina* (West.) Arx.

D. quercina [teleomorph: *Apiognomonina querci-na* (Kleb.) Höhn has of late been frequently isolated from *Q. cerris*, on which it causes dieback of intermediate and apical twigs (Ragazzi *et al.*, 1999a; Ragazzi *et al.*, 1999b).

Materials and methods

Sampling and method of isolation

Three healthy and three declining *Q. cerris* trees, about 20 years old, growing in a pure stand in Val d'Era near the locality of Ulignano (Pisa) at 360–400 m a.s.l. were sampled in April (bud break), June (full vegetation) and October (leaf fall) in 1999 and 2000. Trees in decline were scored on a disease scale of 1 (slight severity = 11–25% defoliating class) or 2 (medium severity = 26–60% defoliating class) in accordance with international normative (Ferretti, 1994).

Twenty current-year twigs, 20 buds and 10 leaves were harvested from each tree. From the wood of each current-year twig 5 cross sections approx. 5 mm in diameter were cut giving 300 sections from healthy and 300 from declining trees. After removal of the scales, two fragments from the buds of the current year were excised, giving 120 fragments from healthy and 120 from declining trees; and from the leaves 5 fragments per leaf of approx. 5 mm² each were cut, giving 150 fragments from healthy and 150 from declining trees.

Each sample was sterilised by dipping in 10% H₂O₂ for 15 min, followed by rinsing five times in sterile water. The samples were then dried on sterile filter paper and incubated on potato-dextrose-agar (Difco Laboratories, Detroit, MI, USA) amended with 0.06 g/l streptomycin in 90 mm Petri dishes. Ten fragments were incubated per dish. Isolations on the samples were carried out within 72 h of the end of sample collection. Incubation lasted for 7 days at 20°C in the dark. After incubation for seven days colonies were transferred to 2% w/v malt-extract agar (Difco Laboratories,) and stored at 4°C.

Assessment of isolation frequency

The isolation frequency (IF) of each endophyte taxon was calculated according to the formula $IF = Ni/Nt \times 100$, where Ni is the number of fragments obtained from twigs, buds and leaves from which the fungus was isolated and Nt the total number of seeded fragments.

Differences in IF between sampling dates and between tree organs yielding samples were processed with ANOVA after the percent data had been transformed with ARCSIN.

Identification

Isolates were grouped by their cultural charac-

teristics and identified by their morphological characteristics.

Fungi with an IF of less than 2% were deemed occasional fungi. Identification was with the keys of Von Arx (1987), Sutton (1980), Carmichael *et al.* (1980), Booth (1971) and Gams (1971).

Results

Fungi were found in all the tree organs, with a frequency varying between organs. From current-year twigs, whether healthy or in decline, the following fungi were isolated: *Acremonium mucronatum* Link., *Acremonium* sp., *Alternaria alternata* (Fr.) Keissler, *Cladosporium cladosporioides* (Fres.) de Vries, *Colpoma quercinum* (Pers. ex St. Am.) Wallr., *Diplodia mutila* Fr. apud Mont., *Discula quercina* (West.) Arx, *Epicoccum nigrum* Link, *Monochaetia* sp., *Phoma cava* Schulzer, *Phomopsis quercina* (Sacc.) Höhn, *Phomopsis* sp. 2, *Phomopsis* sp. 3, *Trichoderma viride* Pers. and *Ulocladium* sp. (Table 1). Current-year twigs had the greatest number of endophytic species. No organ was colonised by all the species found. Buds and leaves from healthy trees harboured only 11 and 6 species, respectively: buds and leaves from declining trees 11 and 6 species each. Fig. 1 shows the IF for

the fungi identified at the species level with an IF of more than 10%, in relation to the health state of the host tree, healthy or declining. Here too, *Diplodia mutila* and *Phomopsis quercina* displayed frequencies greater than 13 and 15% respectively on declining trees, but differences were statistically not significant (error bars overlap).

Table 2 shows that these endophytes were found at all sampling dates though with varying frequency. As regards the IF of individual fungi identified at the species level, at the June samplings (full vegetation), averaging data for 1999 and 2000, *Diplodia mutila* was the most frequent, followed by *Discula quercina*. *Acremonium mucronatum*, *Cladosporium cladosporioides*, *Phomopsis quercina* and *Trichoderma viride* had isolation frequencies ranging from 5.2 to 13.6%.

ANOVA detected highly significant differences ($F \leq 0.01$ and 0.05) between sampling dates ($F \leq 211.12$), between healthy and declining trees ($F \leq 180.45$), and between tree organs examined ($F \leq 90.80$) (Table 3).

Discussion

From the *Q. cerris* trees tested, 15 endophyte fungi were isolated in all. This number is modest

Table 1. Isolation frequency of fungal species from healthy and declining *Quercus cerris* trees in 1999 and 2000. Data are percentages of each fungus over total fungi isolated from that organ summing data of all samplings dates.

Fungal species	Healthy trees			Declining trees		
	Current-year twigs	Buds	Leaves	Current-year twigs	Buds	Leaves
<i>Acremonium mucronatum</i>	7.6	8.7	19.2	10.2	7.7	23.3
<i>Acremonium</i> sp. 2	5.7	5.3	-	7.1	6.3	-
<i>Alternaria alternata</i>	6.0	13.4	22.6	2.2	14.4	24.6
<i>Cladosporium cladosporioides</i>	7.2	5.2	14.2	10.3	4.2	16.6
<i>Colpoma quercinum</i>	8.1	7.5	-	4.0	6.5	-
<i>Diplodia mutila</i>	10.9	7.7	-	13.6	8.5	-
<i>Discula quercina</i>	8.7	7.5	5.1	10.1	7.3	3.4
<i>Epicoccum nigrum</i>	5.2	-	-	1.7	-	-
<i>Monochaetia</i> sp.	6.1	5.6	-	6.9	4.6	-
<i>Phoma cava</i>	5.6	-	-	3.3	-	-
<i>Phomopsis quercina</i>	10.1	8.7	-	15.1	9.7	-
<i>Phomopsis</i> sp. 2	5.3	4.4	-	2.3	3.0	-
<i>Phomopsis</i> sp. 3	4.2	-	-	1.1	-	-
<i>Trichoderma viride</i>	9.3	26.0	20.1	12.1	27.8	18.6
<i>Ulocladium</i> sp.	-	-	18.8	-	-	13.5

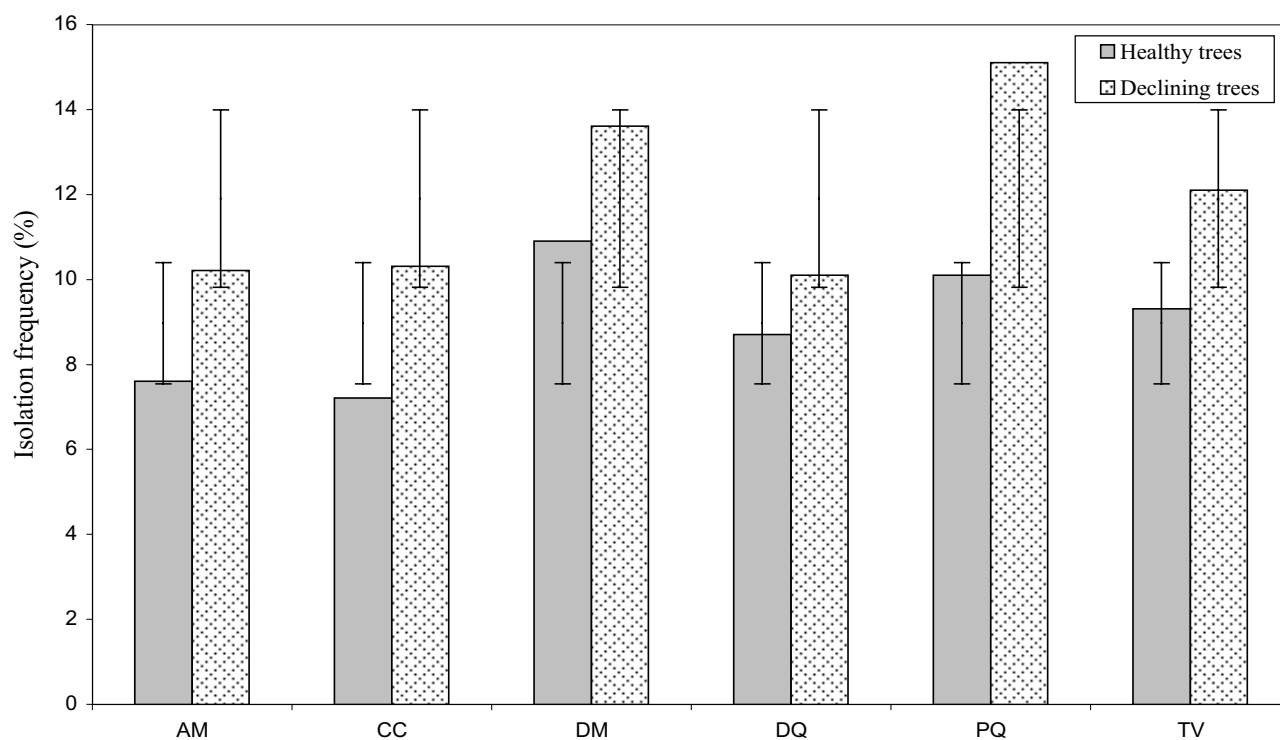


Fig. 1. Isolation frequency (%) of fungal species identified on healthy and declining trees (sum of all samples). The bars indicate the standard deviation. AM = *Acremonium mucronatum*; CC = *Cladosporium cladosporioides*; DM = *Diplodia mutila*; DQ = *Discula quercina*; PQ = *Phomopsis quercina*; TV = *Trichoderma viride*.

Table 2. Isolation frequency of fungal species from healthy and declining *Quercus cerris* trees at three sampling dates in 1999 and 2000. Data are percentages of each fungus over total fungi isolated from that organ summing data of all sampling dates.

	Healthy trees			Declining trees		
	April	June	October	April	June	October
<i>Acremonium mucronatum</i>	8.6	10.6	11.0	10.9	11.1	13.6
<i>Acremonium</i> sp. 2	6.3	2.7	5.7	6.2	2.0	7.3
<i>Alternaria alternata</i>	3.4	2.6	3.4	3.0	2.3	3.8
<i>Cladosporium cladosporioides</i>	8.9	8.8	6.0	9.6	11.4	7.9
<i>Colpoma quercinum</i>	3.6	4.0	5.3	2.1	4.0	5.7
<i>Diplodia mutila</i>	9.2	13.3	6.2	14.4	14.2	9.1
<i>Discula quercina</i>	9.0	12.3	6.7	13.2	12.1	8.9
<i>Epicoccum nigrum</i>	3.6	1.7	3.4	1.9	2.1	3.3
<i>Monochaetia</i> sp.	5.8	4.3	5.3	7.5	1.3	4.3
<i>Phoma cava</i>	5.6	2.6	3.2	2.1	1.9	6.0
<i>Phomopsis quercina</i>	8.7	12.1	5.3	9.2	13.0	7.9
<i>Phomopsis</i> sp. 2	6.3	9.0	4.3	5.0	8.1	5.4
<i>Phomopsis</i> sp. 3	6.2	5.4	4.3	5.1	8.1	5.8
<i>Trichoderma viride</i>	10.5	8.0	5.2	6.7	5.2	5.9
<i>Ulocladium</i> sp.	4.3	2.6	24.7	3.1	3.2	5.1

Table 3. Analysis of variance on the isolation frequency as a function of various parameters (ARCSIN data).

Variation	df	Deviance	Variance	F
Total	9	1915.570		
Between sampling dates	2	1026.044	513.022	211.12 **
Between healthy and declining trees	1	438.518	438.518	180.46 **
Between organs	2	441.288	220.644	90.80 **
Error	4	9.720	2.430	

** $F \leq 0.01$.

when compared with that on other trees (Petrini, 1986; Petrini and Fisher, 1990; Shamon and Sieber, 2000). All the fungi occurred in both healthy and declining trees although with different frequencies in the two health categories.

For current-year twigs of the declining trees, woody tissue was colonised in a range of values from 1.7 to 15.1%. The literature shows: 9.2% for *Q. robur* L., 7.5% for *Fagus sylvatica* L., and 18.6% for *Fraxinus excelsior* L. (Kowalski and Kehr, 1997).

By contrast, the IF from the current-year twigs (woody tissue) was higher than that from the buds or leaves. For example, the IF of *Discula quercina* from woody tissue on healthy trees was 8.7%, compared with 7.5% from the buds and 5.1% from the leaves. On declining trees the corresponding figures were 10.1, 7.3 and 3.4%, respectively on current-year twigs, buds and leaves. Current-year twigs yielded the greatest number of endophytic species: 15, compared with 11 from the buds and 6 from the leaves.

Organ specificity for endophytic fungi has been amply demonstrated on wheat by Sieber (1985), and on Norway spruce and silver fir by Sieber (1988 and 1989).

At the same time a given organ of plants living in different environments, can be colonised by different endophytes: this can be explained as a form of organ specificity related to particular ecological/physiological conditions (Fisher *et al.*, 1991).

In this study twig diameter was correlated with IF. The IF for *D. mutila*, *D. quercina* and *P. quercina* were higher than 15% on healthy trees and 25% on declining trees when the twig diameter was less than 2 cm (current-year twigs), but 7% on healthy trees and 16% on declining trees for twigs with a diameter between 2 and 5 cm (data not shown). This is consistent with the data reported in the

literature for *Q. robur* and *F. sylvatica* (Kowalski and Kehr, 1997).

All fungi found colonised both healthy and declining trees, i.e. they were able, independently of the health state of the tissues, to colonise healthy wood tissue and to adapt themselves saprophytically to degraded tissue.

Other studies have found that many endophytes occur in the xylem of dead or declining trees (Butin and Kowalski, 1983a, 1983b and 1990; Kowalski and Butin, 1989).

Among endophytic fungi with known pathogenic action, *Diplodia mutila* occurs only on current-year twigs (Ragazzi *et al.*, 1997), *Discula quercina* on all tree organs (Ragazzi *et al.*, 1999a and 1999b), and *Phomopsis quercina* only on twigs and buds (Ragazzi, 1991).

These endophytes were isolated at all sampling dates, though the IF differed between dates: it was highest in June (full vegetation). Variations in the IF during the growing season were also reported with *Discula* sp. and an unidentified sterile fungus on Japanese beech (*Fagus crenata* Blume) by Sahashi *et al.*, 1999; with *Apiognomonia errabunda* (Rob.) Höhn. on *Fagus sylvatica* by Sieber & Hugentobler (1987), and with *D. quercina* on *Quercus garryana* Dougl. by Wilson and Carroll (1994). These researchers found *D. quercina* on the leaves, bark, acorns and cotyledons, but only rarely in the wood. Except for this last finding regarding its rare presence in *Q. garryana*, *D. quercina* showed the same IF distribution in our study on *Q. cerris*. With *Discula* sp. on *F. crenata* the IF peaked between 26 May and 11 July, when the trees were in full vegetation, after which it declined on 8 August and remained very low until the end of September (Sahashi *et al.*, 1999).

In this study IF values were very low in the

October (leaf fall) sampling, possibly as a result of hot and dry weather in July and August.

Acromonium mucronatum, a known antagonist of *D. mutila* (Ragazzi et al., 1996) was found on all tree organs, more frequently on declining trees (13.7%) than on healthy trees (11.8%) and at all sampling dates, though its IF was highest in June (full vegetation).

In conclusion, *Diplodia mutila*, *Discula quercina* and *Phomopsis quercina*, three of the most common endophytic pathogens on healthy, but even more on declining *Q. cerris*, *Q. robur* L., *Q. pubescens* Willd., *Q. frainetto* Ten. (Ragazzi et al., 1999a) and *Q. suber* L. (Franceschini et al., 1993) were shown to colonise various tree organs as both parasites and saprophytes over an extended growing period, and therefore must be considered in Italy inciting and contributing factors in the death of oak stands from decline.

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